IN VITRO METHODS TO MEASURE TOXICITY OF CHEMICALS

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ABSTRACT

Rapid screening of industrial compounds for toxicity will require high-throughput *in vitro* assays with which to select candidate compounds for more intensive animal testing. The purpose of this study, sponsored by the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the European Centre for the Validation of Alternative Methods (ECVAM), is to evaluate the utility of *in vitro* cytotoxicity assays for estimating the starting dose for the rat oral acute toxicity test, thus reducing and refining the use of animals in the toxicological assessment of industrial chemicals of military interest. The three-phase study will evaluate 72 coded chemicals with well-defined *in vivo* acute toxicity data, representing a wide range of toxicity and use categories for their ability to induce cytotoxicity as determined by neutral red uptake (NRU) in two cultured cell systems [mouse fibroblast (BALB/c) 3T3 and normal human keratinocytes (NHK)]. Phase I and II studies have been completed under GLP (Good Laboratory Practice) – compliance and the data are presented here.

1. INTRODUCTION

Acute oral toxicity testing in animals has been used since the 1920s to establish the risk of human exposure to various substances. Although methods other than the classical LD_{50} can be used to reduce the number of animals, acute systemic toxicity testing still represents the largest use of animals in safety and other toxicological evaluations. The International Workshop on *In Vitro* Methods for Assessing Acute Systemic Toxicity met in October of 2000 to discuss current *in vitro* methods to reduce and refine the use of animals in toxicity testing. One workshop recommendation was to publish guidance for using *in vitro* basal cytotoxicity assays to estimate starting doses for acute oral lethality tests since these assays were considered sufficiently reliable for this purpose.

The NRU cytotoxicity assay is a cell survival/viability chemosensitivity assay based on the ability of viable cells to incorporate and bind neutral red (NR), a supravital dye. Healthy mammalian cells, when maintained in culture, continuously divide and multiply over time. A toxic chemical, regardless of site or mechanism of action, will interfere with this process and result in a reduction of the growth rate as reflected by cell number. Cytotoxicity is expressed as a concentration dependent reduction of the uptake of the NR after chemical exposure, thus providing a sensitive, integrated signal of both cell integrity and growth inhibition. Alterations of the cell surface or sensitive lysosomal membranes lead to lysosomal fragility and other changes that gradually become irreversible. Such changes brought about by the action of xenobiotics result in a decreased uptake and binding of NR. It is thus possible to distinguish between viable, damaged, or dead cells. The ability of each chemical to reduce cell growth or inhibit NR uptake by 20%, 50%, and 80% is measured by the corresponding concentration (i.e., ICx) and compared with toxicity data from *in vivo* rodent studies from the Registry of Cytotoxicity (RC) developed by ZEBET (German Center for the Documentation and Validation of Alternative Methods).

2. MATERIALS AND METHODS

2.1 The Neutral Red Uptake (NRU) Cytotoxicity Assay

Two neutral red uptake assays were conducted in Mouse fibroblast (BALB/c) 3T3 and Normal Human Keratinocyte (NHK). Cell cultures were grown in 96-well microtiter plates and exposed to test chemical. After 48 hrs incubation, the test chemical was removed and NR solution was applied to the cells. The cells were incubated again, the excess NR solution was removed, and NR was eluted from the cells. The NRU was determined by using a microtiter plate reader/spectrophotometer to measure the optical density (at wavelength 540 ± 10 nm) of the eluted NR dye in the 96-well plate. A calculation of cell viability expressed as NRU was made for each concentration of the test chemical by using the mean NRU of six replicate values (minimum of four acceptable replicate wells) per test concentration. The cell viability value was compared with the mean NRU of all vehicle control (VC) values (provided VC values have met the VC acceptance criteria). Relative cell viability was then expressed as percent of untreated VC.

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2.2 Chemicals

Chemical selection was based on the following criteria:

- Chemicals representative of five Globally Harmonized System (GHS) categories of acute toxicity (OECD 2001) as well as unclassified chemicals
- Chemicals representative of those regulated by various regulatory authorities.
- Chemicals that have acute oral rodent toxicity data available
- Chemicals that have acute oral human toxicity data available and/or have human exposure potential

Chemicals were coded, packaged and shipped to the participating laboratories by an independent laboratory.

2.3 Study Conduct

The validation study was conducted in compliance with Good Laboratory Practice (GLP) Standards.

3. RESULTS

3.1 Chemical selection

Table 1. Chemicals tested in phases Ib and II

Chemical	CAS	RC	MEIC	Observed – Predicted log LD_{50}[1]	Corrosive	Volatile
$LD_{50} \leq 5$ mg/kg						
Aminopterin	54-62-6	3	NA	-0.652	No	No
Sodium selenate	13410-01-0	NA	NA	NA	No	No
$LD_{50} > 5 - \leq 50$ mg/kg						
Colchicine	64-86-8	6	60	-0.593	No	No
Arsenic trioxide	1327-53-3	153	26	-0.591	No	No
$LD_{50} > 50 - \leq 300$ mg/kg						
Sodium I fluoride	7681-49-4	106	14	-0.109	No	No
Cadmium chloride	10108-64-2	81	NA	-0.336	No	No
$LD_{50} > 300 - \leq 2000$ mg/kg						
DL-Propranolol HCl	350-60-90	54	23	-0.023	No	No
Lithium carbonate	544-13-2	327	20	-0.256	No	No
$LD_{50} > 2000 - \leq 5000$ mg/kg						
Potassium chloride	7447-40-7	346	50	0.085	No	No
Chloramphenicol	56-75-7	91	45	0.441	No	No
$LD_{50} > 5000$ mg/kg						
2-Propanol	67-63-0	128	10	0.396	No	Yes
Ethylene glycol	107-21-1	360	7	0.321	No	No

Notes: [1]Available only for chemicals included in the RC; NA – not applicable.

[2] MEIC – the international MultiCenter Evaluation of *In Vitro* Cytotoxicity

3.2 Cytotoxicity of 12 Tested Chemicals

Figure 1 illustrates the IC50 values for 12 coded chemicals collected from the three laboratories participated in this validation study. Top panel shows 3T3 NRU results. Bottom panel shows NHK NRU results. Error bars show standard deviation. Numbers above error bars are intralaboratory coefficient of variation (CV). The mean intralab CV was 23% for both assays. The interlab CV for the 3T3 assay was 40% and that for the NHK assay was 25%. Due to the relative insolubility of lithium carbonate in the 3T3 medium, only one laboratory obtained IC_{50} data for that assay. Random effects analysis of variance (ANOVA) shows no significant difference among the laboratories at $p< 0.05$; however, analyses for the following chemicals/assays were not calculable: colchicine, potassium chloride, and propranolol HCl for the 3T3 assay; and cadmium chloride, chloramphenicol, and potassium chloride for the NHK assay.

3.3 Comparison of the results of the two assays and three labs performances

IC_{50} results are graphed with LD_{50} values used in the RC as a Figure 2. Top panel shows 3T3 NRU results while bottom panel shows NHK NRU results. Bold line shows RC regression. Lighter lines show RC prediction interval. Sodium selenate is not shown because it was not included in the RC. Comparison of the regressions is shown in Table 2. The comparison of slopes and intercepts showed that no lab or assay specific results were significantly different from the RC regression.

4. DISCUSSION

A primary goal of this validation study is to evaluate the usefulness and effectiveness of *in vitro* cytotoxicity assays for reducing and refining animal use for acute oral toxicity determinations of chemicals by predicting starting doses for *in vivo* rodent lethality assays. The approach for predicting starting doses for acute oral lethality tests, described by the Guidance Document (ICCVAM 2001b) is based on the linear regression analysis of rodent oral LD_{50} and *in vitro* IC_{50} values for 347 chemicals in the Registry of Cytotoxicity (RC) developed by ZEBET (German Center for the Documentation and Validation of Alternative Methods) (Halle, 1998) Figure 3 shows the prediction model evaluated by the Registry of Cytotoxicity (RC) regression between cytotoxicity values (IC_{50}) and rodent acute oral LD_{50} values of 347 chemicals. The middle line represents the fit of the data to a linear regression ($r=0.67$). The two additional lines represent the empirical boundaries of the prediction interval (± log 5). The equation of the regression line is: $\log LD_{50} = 0.435 \times \log IC_{50X} + 0.625$

The feasibility of developing a preliminary human prediction model will be evaluated by using the *in vitro* results, obtained in both tests, for the 12 chemicals tested in Phase I and II, and the corresponding human sublethal and lethal blood concentrations (MEMO database; Ekwall *et al*. 1998). *In vitro* data for Phase III Chemicals will be used to validate the model.

This validation study is a three phases study. Phases I study consisted two parts, e.g., Ia and Ib. Phase Ia focused on the toxicity of the positive control (PC) chemical, sodium laurel sulfate, and established the historical data base for the control for both assays. Phase Ib and II characterized the toxicity of 12 test chemicals and the results presented in this paper. The data show that 1) the *in vitro* IC_{50} values are consistent with the RC model for prediction of rodent acute toxicity; 2) the two assays have good intralaboratory reproducibility, indicating the feasibility for extending their use to prediction of starting doses for *in vivo* acute oral lethality testing. This validation study has demonstrated that these two *in vitro* assays are useful biotechniques for quickly screening and determining toxicity of chemicals of interest to industry, environment, and defense.

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Study Management Team: National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM), and ECVAM.

Project Advisory Team: German Center for the Documentation and Validation of Alternative Methods (ZEBET at BgVV), Acute Toxicity Working Group (ATWG), The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), European Chemicals Bureau (ECB) and BioReliance Corp., Rockville, MD USA

Other Colleagues: Institute for In Vitro Sciences, Rockville, MD and (laboratory 2) and University of Nottingham, UK (laboratory 1)

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U.S. Army Edgewood Chemical & Biological Center (ECBC)Molecular Engineering Team (MET) (2002): STANDARD OPERATING PROCEDURE (SOP) – DOCUMENT: NHK **02**: The Normal Human Keratinocyte (NHK) Neutral Red Uptake Cytotoxicity Test - A Test for Basal Cytotoxicity for an *In Vitro* Validation Study.

U.S. Army Edgewood Chemical & Biological Center (ECBC) Molecular EngineeringTeam (MET) (2002): STANDAR OPERATING PROCEDURES (SOPs) for Good Laboratory Practices (GLP).

CONCLUSION

- Intralaboratory reproducibility was the same for both assays (mean CV= 23%), but interlaboratory reproducibility was better for the NHK assay (mean CV = 25% vs 40% for 3T3).

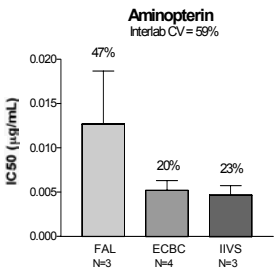
- Judging by goodness of fit r^2 values, the 3T3 assay provided a better linear regression (i.e., IC_{50} was a better predictor of LD_{50}) (see Table 2). This may be due to the five fold difference in the sensitivity of NHK cells to aminopterin (see Figure 2).

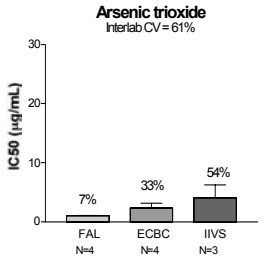
- Phases Ib and II of the validation study confirmed that cytotoxicity results from individual cytotoxicity assays do not differ from the RC regression (see Table 2). Phase III results will determine how many animals may be saved using this approach for estimating starting doses for LD_{50} tests.

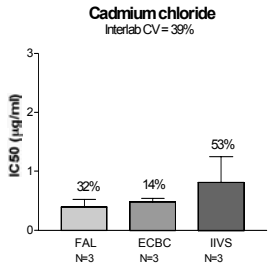
- Based on these two phase studies phase III study has been advanced in which the two methods developed from these studies has been used to measure cytotoxicity of 60 selected chemicals.

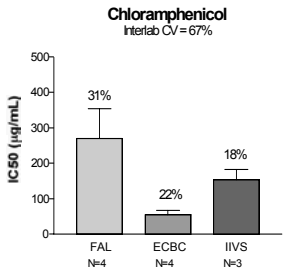
- The two *in vitro* methods will be very useful for rapid screening of the toxicities caused by diverse industrial chemicals of military interest.

3T3 NRU

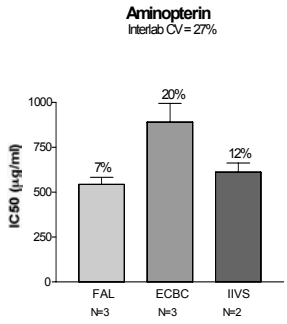


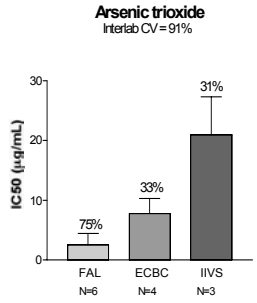


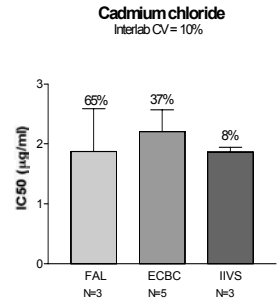


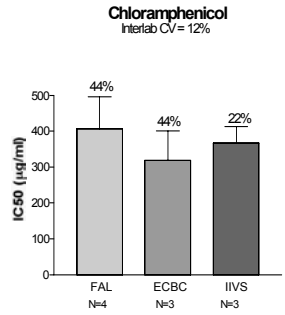


NHK NRU









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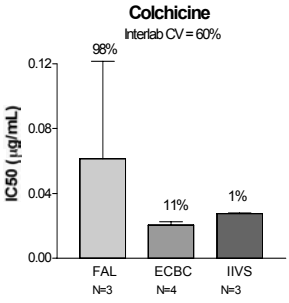
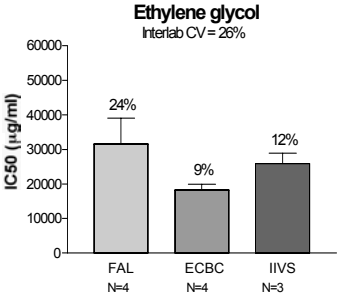
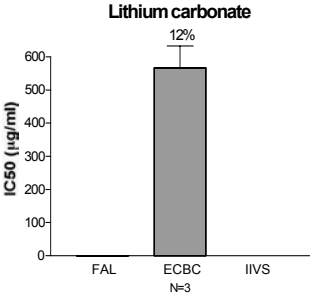
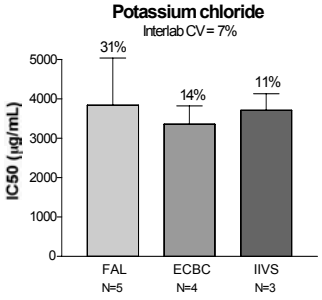
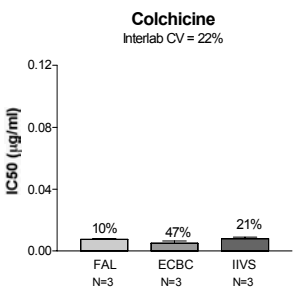


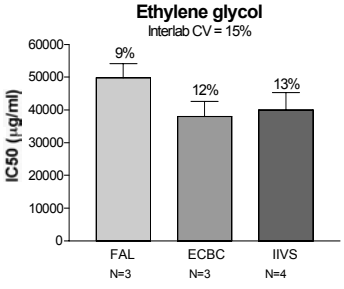


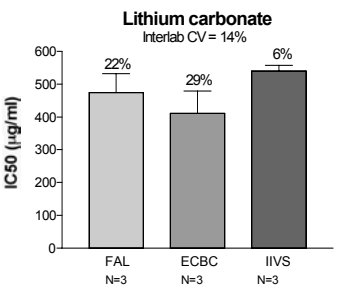


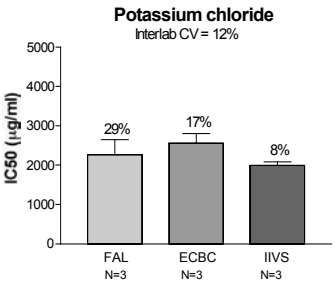


NHK NRU

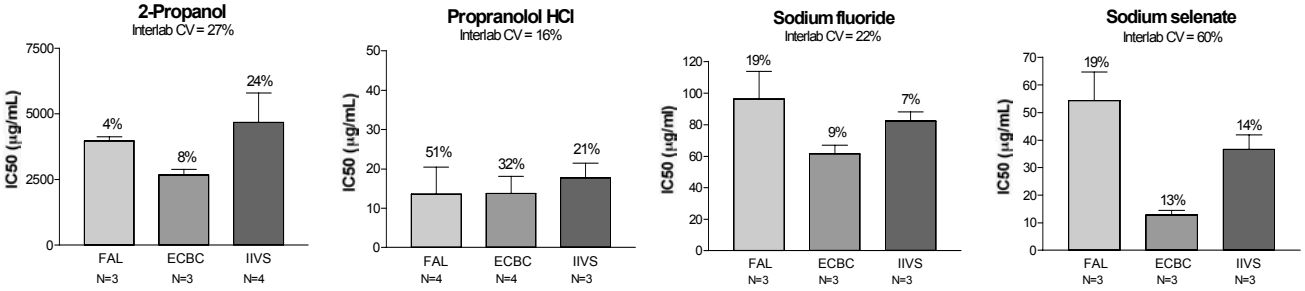








3T3 NRU



NHK NRU

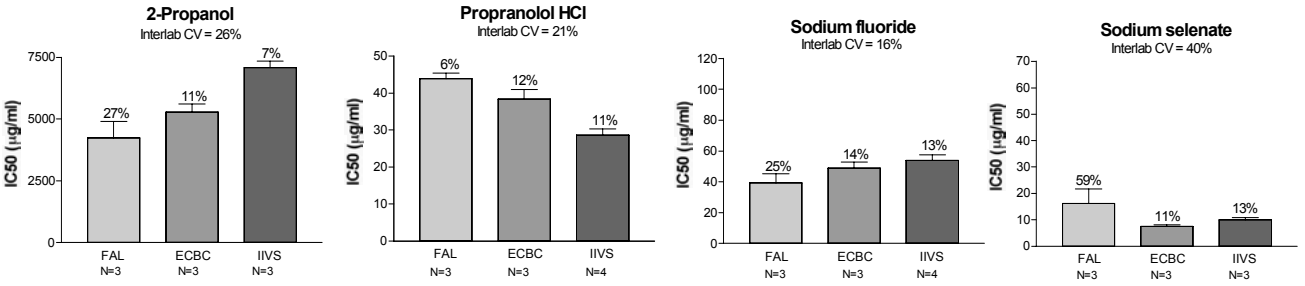


Figure 1. Phases Ib and II preliminary results: IC50 values for 12 coded chemicals.

Labs: FAL – FRAME Alternatives, University of Nottingham, UK; ECBC – US Army Edgewood Chemical Biological Center, MD, USA; IIVS – Institute for In Vitro Sciences, Rockville, MD, USA.

Table 2. Comparison of lab/assay results with RC regression

Regression	Slope	y intercept	r^2	Comparison with RC	
				Slopes (p-value)	Intercepts (p-value)
RC	0.435	0.625	0.4519	Not applicable	Not applicable
FAL 3T3	0.592	0.722	0.958	0.114	0.899
FAL NHK	0.545	0.402	0.619	0.327	0.305
ECBC 3T3	0.579	0.771	0.937	0.134	0.883
ECBC NHK	0.525	0.383	0.576	0.422	0.254
IIVS 3T3	0.582	0.706	0.949	0.132	0.875
IIVS NHK	0.545	0.357	0.588	0.341	0.211

Labs: FAL – FRAME Alternatives, University of Nottingham, UK; ECBC – US Army Edgewood Chemical Biological Center, MD, USA; IIVS – Institute for In Vitro Sciences, Rockville, MD, USA.

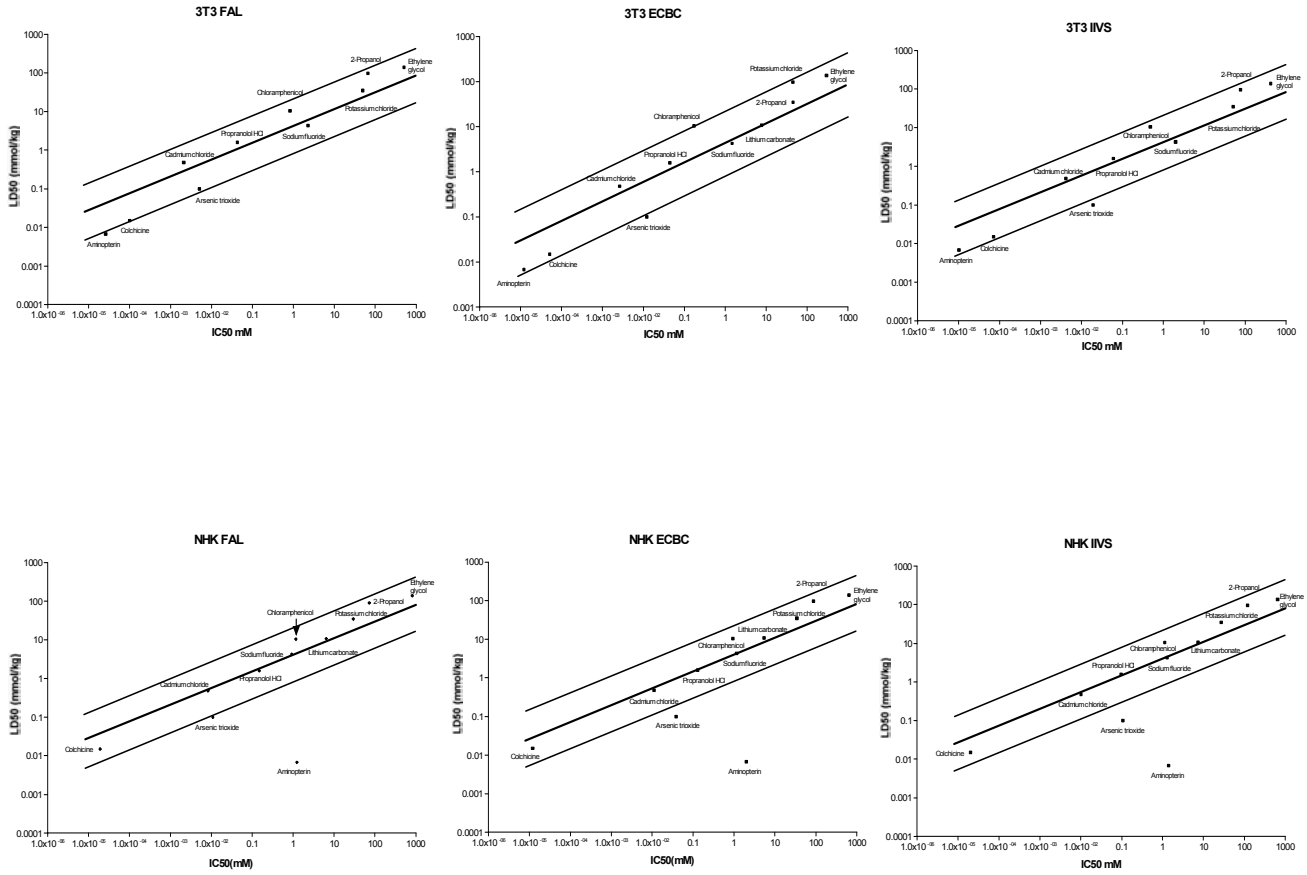


Figure 2. Phase Ib and II preliminary IC_{50} values on RC regression

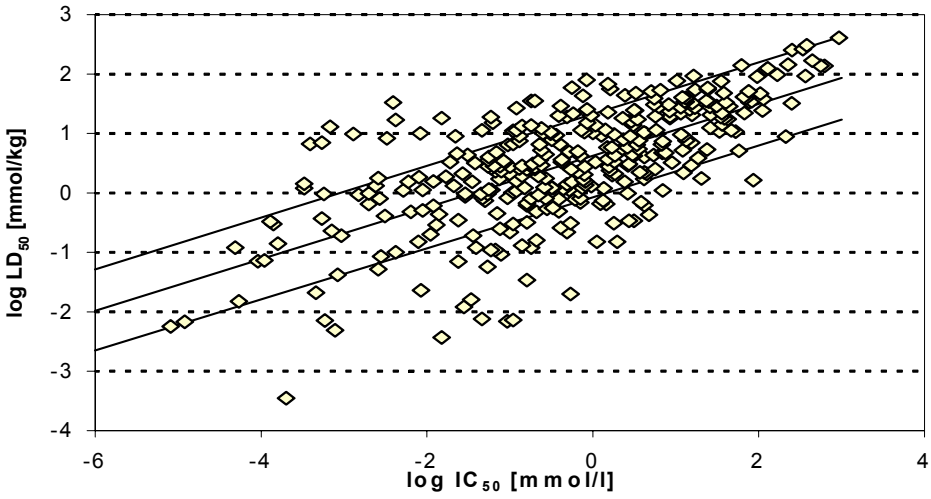


Figure 3. Registry of cytotoxicity regression between cytotoxicity (IC_{50x}) and rodent acute oral LD_{50} values for 347 chemicals.